Random Mutagenesis and Selection for RubisCO Function in the Photosynthetic Bacterium *Rhodobacter capsulatus*

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By Richa Patel

The Ohio State University

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Project Advisors: Dr. F. Robert Tabita, Professor
Dr. Sriram Satagopan, Postdoctoral Researcher
Department of Microbiology

ABSTRACT

Ribulose 1, 5-bisphosphate carboxylase/oxygenase (RubisCO) catalyzes a reaction of fundamental importance, the incorporation and reduction of atmospheric carbon dioxide into organic matter. This enables organisms to grow autotrophically, that is at the expense of CO_2 as the sole carbon source. Despite its key role, all RubisCO enzymes found in nature are plagued by their susceptibility to inhibition by oxygen and are among the most sluggish biological catalysts found in nature. Based on the sequence of its component amino acids, RubisCO has been classified into four molecular forms: form I, form II, form III, and form IV (RubisCO-like proteins, RLPs). Whereas enzyme forms I, II and III catalyze CO_2 fixation, the form IV proteins cannot perform this function, presumably due to the absence of appropriate active-site residues. Substitutions were made in the *Cholorobium tepidum* RLP (CtRLP) active-site residues to reflect their identities in bonafide RubisCOs (Mut*)* but they had no effect on the RLP's ability to function as a RubisCO. Additional secondary structural changes had also been introduced in the CtRLP by deleting a CD loop that is absent in RubisCOs and by inserting a β-hairpin structure that is absent in RLPs (MutCDΔβH). Yet after all the changes were introduced, no RubisCO activity was observed (1). Thus, as a part of this research thesis, the DNA encoding MutCDΔβH was randomly mutagenized and bioselection was carried out in *Rhodobacter capsulatus*, SB I/II$^-$, a RubisCO knockout strain, to select for mutations that may complement for RubisCO activity.

Apart from RLPs that are completely devoid of RubisCO activity, there are functional differences even among forms I, II and III. Upon sequence comparisons, residue 165 of *Rhodopsuedomonas palustris* form II RubisCO was identified as a unique active-site residue that was divergent among the three forms of RubisCO (threonine in form I, isoleucine in form II and valine in form III). In order to examine the importance of this residue for RubisCO function, it was substituted with an alanine (a neutral substitution), threonine (resembling form I RubisCO) or a valine (resembling form III RubisCO) in the form II RubisCO (*cbbM*) from *R. palustris*. To assess the functional competency of these mutants, they were complemented in the RubisCO knock-out strain of *R. capsulatus*, strain SB I/II$^-$. Whereas the I165A and I165T mutants were unable to support growth under photoautotrophic conditions, the I165V enzyme could. To identify other regions in the protein structure that could complement the negative phenotypes conferred by I165A and the form-I-like I165T substitutions, random mutagenesis and bioselection in *R. capsulatus* SBI/II$^-$ was performed with I165A and I165T mutant genes. It has been possible to identify an A165V substitution as a pseudo-revertant of the original I165A mutant. However, it was not possible to identify other potential suppressor mutations. A problem was identified with the methodology that could likely interfere with the efficient recovery of suppressor mutations. Once this problem is resolved, the suppressor recovery and analysis will likely reveal other residues that play a vital role in the function of the enzyme.

BACKGROUND

The enzyme ribulose 1, 5 bisphosphate carboxylase/oxygenase (RubisCO) is the most abundant protein found on earth. This enzyme catalyzes CO_2 fixation in the Calvin-Benson-Bassham (CBB) cycle (Figure 1) (2). The CBB pathway thus allows atmospheric carbon dioxide to be incorporated into organic matter which is absolutely essential for the survival of plants and many environmentally significant microorganisms. However, limitations of RubisCO catalytic activity due to competition between O_2 and CO_2 at the same active-site make it an inefficient enzyme (3). Hence, efforts have been made in order to engineer an improved RubisCO enzyme that can fix carbon more efficiently (1). RubisCO molecules with improved carbon fixation can ultimately contribute to meeting the increasing food and energy requirements for both plants and animals. Carbon compounds derived from the fixed CO_2 are prevalent within the animal body, in everyday clothing, and in all organic products encountered in day-to-day life. In addition, RubisCO catalysis provides the major means by which atmospheric CO_2 may be removed from the biosphere, of crucial importance as we face the consequences of global warming.

The CBB pathway is a reductive pentose phosphate pathway carried out by photoautotrophic and chemoautotrophic bacteria, plants and algae. RubisCO binds ribulose 1,5-bisphosphate (RuBP) during the first and rate-limiting step of the pathway. RuBP, the substrate for RubisCO, is then de-protonated at C-2 to form a 2,3 enediol(ate) which is attacked by either CO_2 or O_2. Attack by CO_2 results in the production of two molecules of 3-phosphoglycerate (PGA), which is then ultimately channeled to sugars

and then other carbon skeletons (Figure 1) (2). On the other hand, O_2 binding results in the production of one molecule of 3-phosphoglycerate and one molecule of 2-phosphoglycolate (2-PG). Thus, when RuBP reacts with oxygen there is less carbon that is incorporated into the cell's biomass and 2-PG metabolism also leads to CO_2 release from the cell (3).

RubisCO has been classified into four molecular forms: form I, which is present in many different types of bacteria, algae and in all plants; form II, which is prevalent mostly in some types of bacteria; form III, which is found only in archaea; and form IV (RLPs) that were recently discovered in both photosynthetic and non-photosynthetic bacteria, archaea, and some algae (4). Despite structural differences observed in bonafide RubisCOs (forms I, II and III), their active-site residues and reaction mechanisms are highly conserved and they all catalyze CO_2 fixation, unlike form IV proteins (RLPs) (3).

The genome of the nonsulfur, purple bacterium *Rhodobacter capsulatus* encodes form I and form II RubisCO enzymes. *R. capsulatus* strain SBI/II$^-$ is a previously constructed strain with both RubisCO forms deleted and hence, is unable to carry out CO_2-dependent growth (5). Thus, *R. capsulatus* SBI/II$^-$ offers a facile host for selecting functional RubisCO (3). Studies described in this thesis are based on the use of the *R. capsulatus* strain SB I/II$^-$ for "bioselection" of RubisCO function from randomly mutagenized RLP and mutant RubisCO genes.

The RLP-encoding gene utilized in this study was obtained from the green sulfur bacterium, *Chlorobium tepidum*. *C. tepidum* RLP (CtRLP) is unable to catalyze the CO_2 fixation reaction but has been shown to be involved with sulfur metabolism and response to oxidative stress. CtRLP has a potential evolutionary link to bona fide RubisCOs (forms

I, II and III) (6). Therefore, further analysis of CtRLP has been carried out to gain a better understanding of the evolution of the RubisCO active-site.

R. palustris is a purple non-sulfur phototrophic bacterium whose genome also encodes for two forms of RubisCOs, i.e., form I and form II. However, much interest has been drawn to the *R. pa*lustris form II RubisCO because its quaternary structure does not resemble the only other known structure of a functionally comparable form II RubisCO; i.e., from *Rhodospirillum rubrum*. *R. rubrum* RubisCO is a dimer (L_2), whereas *R. palustris* form II RubisCO is a hexamer (L_6) (7). Thus, initial structural studies suggested that the form II RubisCO of *R. palustris* may be a good candidate for analysis of RubisCO structure-function relationships. A comparison of the active-site residues among the different RubisCO forms indicated that the identity of residue 165 of *R. palustris* form II RubisCO (or the equivalent residue from other forms) was conserved among all the form II enzymes, but was divergent among the other two forms of bonafide RubisCOs (threonine in form I, isoleucine in form II and valine in form III) (1). Part of the study performed here was thus directed at determining the importance of this residue.

METHODS AND MATERIALS

Generation of mutations: The *C. tepidum* RLP gene containing substitutions at 12 residues and additional structural changes at two other sites (MutCDΔβH) had been constructed earlier via site-directed mutagenesis and gene insertion/deletion (8). Likewise, mutations at residue 165 of the *R. palustris* form II RubisCO gene (*cbbM*) were generated by site-directed mutagenesis (1). These mutant genes, all of which had been

cloned into the pCR2.1-TOPO vector (Invitrogen), were used as templates for error-prone PCR mutagenesis with M13 forward and reverse primers. These primers amplified the template genes along with the flanking restriction-enzyme sites for *Xba*I and *Sac*I endonucleases.

The 50 µl PCR reaction mixture contained 75 ng of template DNA, 1 µl of each primer at a concentration of 10 µM, 1 µl of 25µM dNTPs, 10X PCR buffer, 2 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM $MnCl_2$, 1 µl *Taq* DNA polymerase (5 U) (Invitrogen). $MnCl_2$ plays the role of lowering the fidelity of *Taq* polymerase and allows the introduction of a limited number of mutations into the genes. The PCR reaction was run in a MJ Mini Personal Thermal Cycler and the cycling parameters included an initial 2-min denaturation step at 94°C, followed by 35 cycles each containing a 1-min denaturation at 94°C, 1-min annealing at 56°C and a 2-min extension at 72°C. The profile was completed with a 10-min final extension step at 72°C, and then the products were stored at 4°C until further analysis.

After amplification, the products were separated according to size on 1% agarose gels and stained with ethidium bromide for analysis in a UV-light transilluminator. The PCR products were purified using a PCR Purification Kit (Qiagen) and were digested with the *Xba*I and *Sac*I endonucleases (New England Biolabs). The digests were cloned into pRPS-MCS3 and transformed into chemically competent *Esherichia coli* Top10 cells (Invitrogen) (3). The transformed cells were grown on Luria-Bertani (LB) plates (10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride, 15 g/l agar, pH adjusted to 7.5) with tetracycline (12.5 µg/ml), X-gal (20 mg/ml in DMF) and IPTG (0.5 M). If the *E. coli* cells took up the gene of interest it would have disrupted the *lacZ* gene (encoding β-

galactamase) in the plasmid, yielding a white colony. Hence, all white colonies seen after incubation for 24 h at 37 °C were harvested.

Mating Procedure: *R. capsulatus* SBI/II$^-$ was grown in CSOC media (6 g/l peptone, 5 g/l yeast extract, 10 mM NaCl, 3 mM KCl, , 0.1 μg/ml biotin, 2 mM Ormerod's basal salts and 1 μg/ml thiamine and niacin), kanamycin (5 μg/ml) and spectinomycin (10 μg/ml) for 48 h at 30°C. The *E. coli* 2013 cells, carrying the plasmid that facilitates bacterial conjugation (3), were grown in LB media (10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride, pH adjusted to 7.5) containing kanamycin (25 mg/ml) for 24 h at 37°C.

The harvested cells carrying the PCR-mutagenized inserts in the pRPS-MCS3 vector were washed and mated along with the helper strain *E. coli* 2013 and the *R. capsulatus* strain SBI/II$^-$ (3). The mating mixture was incubated for 24 h at 30°C on peptone yeast extract (PYE) plates (3 g/l peptone, 3 g/l yeast extract, 10% Ormerod's basal salts, 1 μg/ml thiamine and niacin, 0.1 μg/ml biotin, and 15 g/l agar). The mating mixture was then incubated for 48 h at 30°C on PYE plates (3 g/l peptone, 3 g/l yeast extract, 10% Ormerod's basal salts, 1 μg/ml thiamine and niacin, 0.1 μg/ml biotin, and 15 g/l agar), containing rifampicin (100 μg/ml) and tetracycline (2 μg/ml) to ensure that the mating procedure was successful.

Transconjugants from the PYE-rifampicin-tetracycline plates were washed with minimal media (10% Ormerod's basal salts, 20% $(NH_4)_2SO_4$, 1 μg/ml thiamine and niacin, 0.1 μg/ml biotin and 50 ml/l phosphate buffer) and grown on Ormerod's minimal media plates (10% Ormerod's basal salts, 20% $(NH_4)_2SO_4$, 1 μg/ml thiamine and niacin, 0.1 μg/ml biotin, 50 ml/l phosphate buffer and 20 g/l Gold USB agar). All plates were

incubated for 168 h (one week) at 30°C with a constant light source, a CO_2-generating gas pak to produce a 10% CO_2/90% H_2 atmosphere (Oxoid), and palladium catalyst that scavenges the oxygen. Since no carbon source was provided in the minimal medium, these conditions allowed photoautotrophic growth. The *R. capsulatus* SBI/II$^-$ knockout strain does not support photoautotrophic growth and hence, any growth that was seen on the plates must have resulted from cells that had a mutation in the RLP or *cbbM* gene that conferred positive growth phenotype with respect to CO_2 fixation.

R. capsulatus SBI/II$^-$ complemented with the wild-type *Synechococcus* PCC6301 *rbcLS* gene was used as a positive control for the mating procedure (3). *R. capsulatus* complemented with the *R. palustris* wild-type *cbbM* gene was used as a positive control for photoautotrophic growth supported by a form II RubisCO gene. Additionally, *R. capsulatus* complemented with *R. palustris cbbM* genes containing I165A and I165T substitutions were used as negative controls. Since *R. capsulatus* was complemented with the randomly mutagenized products of the *cbbM* gene with existing I165A or I165T substitutions, a growth phenotype that resembled the positive wild-type *cbbM* control would indicate that the colony contained a suppressor mutation or a reversion.

Selection for mutations: The colonies that grew under photoautotrophic conditions were re-streaked onto Ormerod's minimal media plates. The plates were re-incubated for approximately 168 h (one week) at 30°C with a constant light source in a clear anaerobic jar with an Oxoid CO_2 Generating Pack to produce a 10% CO_2/90% H_2 atmosphere to obtain isolated colonies. During the mating procedure, *R. capsulatus* is capable of taking in more than one pRPS-MCS3 plasmid. A colony isolated after repeated re-streaking would be less likely to contain more than one pRPS-MCS3 plasmid

within it since the plasmids that do not contribute to photoautotrophic growth would be assumed to be diluted out.

To confirm that only a plasmid with the gene of interest was present in the isolated colony the plasmid was purified using a Plasmid Miniprep Kit (Qiagen) and transformed into *E. coli* Top10 cells. The transformants were plated on LB, tetracycline, IPTG and X-gal plates and incubated for 24 h at 37°C. Observation of the plates showed only white colonies indicating that the *R. capsulatus* colony contained the pRPS-MCS3 plasmid with the gene of interest, which conferred the positive growth phenotype. Additionally, the colony was purified and sent for sequencing to further confirm that the plasmid took up the gene of interest.

Identification of suppressor mutations: The colonies observed on minimal media plates were inoculated in a 10-ml CSOC media (6 g/l peptone, 5 g/l yeast extract, 10 mM sodium chloride, 3 mM potassium chloride, 1 μg/ml thiamine and niacin, 0.1 μg/ml Biotin, 10% Ormerod's basal salt) containing kanamycin (5 μg/ml), spectinomycin (10 μg/ml) and tetracycline (2 μg/ml), which would only select for transconjugants. The plasmid was then purified using a Plasmid Miniprep Kit (Qiagen) and sent for sequencing at the Plant Microbe Genomics Facility (PMGF) at The Ohio State University using appropriate primers.

RESULTS AND DISCUSSION

Previous studies by Dr. Satagopan: Prior studies in our laboratory have focused on the RLP encoding gene from *C. tepidum*. Analysis of x-ray crystal structures by

comparison of active-site residues of bona fide RubisCOs and CtRLP has revealed that 10 out of 19 RubisCO active-site residues are different in CtRLP (4, 8). The inability of CtRLP to catalyze the carboxylation reaction is presumably due to the lack of these active-site residues. To test this hypothesis, substitutions were made at the 10 active-site residues and 2 additional residues (Q49T, E119N, V170T, N174K, F288R, I320H, M321S, P322G, R327K, G357S, S359G and R383G), all of which appeared to contribute to the functional differences between bona fide RubisCOs and CtRLP. However, no CO_2 fixation activity could be detected in the mutant enzyme (1, 8). Based on structural analysis, it was of interest to add additional changes to CtRLP so it resembles form I Spinach RubisCO. Hence, a CD loop absent in bona fide RubisCOs was deleted and a β-hairpin structure present in bona fide RubisCOs was introduced into CtRLP (1, 8). These changes only resulted in destabilizing the protein, but the protein was still incompetent with respect to RuBP carboxylation (1). The resultant mutant gene (Mut*CD*Δ*βH*) was inserted into the pRPS-MCS3 expression plasmid (Figure 2) and complemented into *R. capsulatus* SBI/II$^-$ via triparental matings (Figure 3). As expected from the failure to catalyze the RubisCO reaction in vitro (1), the mutant protein was unable to complement *R. capsulatus* strain SB I/II$^-$ for photoautotrophic growth. Because bonafide RubisCOs and CtRLP have additional differences in residues other than the active-site residues, it is possible that other substitutions in either single or multiple amino acids may complement the structural changes for RubisCO function. Therefore, a part of this thesis work was directed towards generating random mutations in the Mut*CD*Δ*βH* gene that could confer photoautotrophic growth.

Additionally, previous studies were also conducted on the form II RubisCO gene from *R. palustris*. A comparison of the active-site residues among the different RubisCO forms indicated that the identity of residue 165 of *R. palustris* form II RubisCO was divergent among the other two forms of bonafide RubisCOs (threonine in form I, isoleucine in form II and valine in form III). To examine the importance of this residue, substitutions had previously been generated by Dr. Satagopan in the form II RubisCO (*cbbM*) from *R. palustris* via site-directed mutagenesis. Isoleucine at residue 165 was substituted to alanine (a neutral substitution), threonine (resembling form I RubisCO) and valine (resembling form III RubisCO) in the *R. palustris* form II RubisCO (*cbbM)* (1). Following site-directed mutagenesis, the mutated *cbbM* genes that encode I165A, I165T and I165V were cloned into pRPS-MCS3 (Figure 2) and complemented into *R. capsulatus* SBI/II$^-$ by triparental matings, as described previously (Figure 3). Upon complementation, it was observed that unlike the I165V the other two mutant enzymes, I165A and I165T, had a detrimental effect on the growth phenotype of *R. palustris* under photoautotrophic conditions. (Figure 4) (1). The growth phenotypes were supported by the kinetic properties of the purified enzymes analyzed in vitro (Table 1) (1). The Michaelis constant (K_m) is the substrate concentration at which the enzyme reaches half its maximum velocity. The K_m (CO_2) values for I165A and I165T enzymes were significantly higher than the K_m (CO_2) values of wild-type *cbbM* and I165V enzymes. The K_m (CO_2) values for I165A and I165V enzymes were 520 ± 96 μM and 697 ± 250 μM, respectively. In comparison, the K_m (CO_2) values for wild-type *cbbM* and I165V enzymes were only 245 ± 64 μM and 300 ± 35 μM, respectively (Table 1) (1). Hence, it is evident that a higher concentration of CO_2 is required for I165A and I165T enzymes to

be saturated with substrate CO_2, which may explain, in part, the detrimental effects these enzymes have on the growth phenotype under photoautotrophic conditions. It was also evident that the K_m (O_2) value for the I165T enzyme was substantially higher (2883 ± 283 μM) than the K_m (O_2) value for wild-type *cbbM* (190 ± 11 μM), which indicates that I165T substitution affects the oxygenation reaction as well. Additionally, the I165A enzyme had a very low specificity constant (6 ± 2) in comparison to the wild-type enzyme specificity constant (12 ± 1), which indicates that the I165A mutant is defective in partitioning CO_2 and O_2. Furthermore, it was observed that the K_m (RuBP) value for I165T enzyme was 38 ± 1 μM, which is somewhat higher than the K_m (RuBP) of wild-type *cbbM*, I165A and I165V enzymes (12 ± 2 μM, 10 ± 2 μM and 29 ± 2 μM, respectively). This indicated that the I165T substitution affected not only the carboxylation/oxygenation reaction, but also the initial step of binding RuBP and proton abstraction to yield the 2,3-enediolate. Therefore, the growth phenotypes conferred by I165A, I165T and I165V enzymes as well as kinetic properties of these enzymes indicate that this residue might indeed be critical for catalysis (1).

Amino-acid substitutions at either residue 165 or second-site suppressors could complement the negative phenotypes of I165A and I165T mutants. By observing the growth phenotypes conferred by these substitutions, a better understanding could be gained of RubisCO structure-function relationships. If second-site suppressors were to be obtained for either I165A and/or I165T mutants, it could potentially result in the identification of additional regions in the protein structure that are important for RubisCO structure and function. Alanine and threonine substitutions at residue 165 were originally created by engineering the codons GCC and ACC, respectively. A single base-pair

mutation within these codons can yield one of many amino acids (Table 2) (1). In an effort to recover suppressors that complement these negative mutants, random mutagenesis and bioselection in *R. capsulatus* SBI/II$^-$ was carried out on the mutant *cbbM* genes that encode the I165A and I165T enzymes. Finally, the growth phenotype was observed under photoautotrophic conditions to identify mutations that suppress either the I165A or I165T enzyme activity.

Current Studies: In this study, the random mutagenesis procedure was utilized with both the mutant CtRLP RLP gene and the mutant form II RubisCO *cbbM* gene previously constructed (1). The PCR mutagenesis protocol was standardized to yield 1-2 single base pair mutations per copy of the amplified gene. Following the mutagenesis procedure, the respective mutated genes were cloned into *E. coli* Top 10 cells which were mated with *R. capsulatus* SBI/II$^-$. The mated cells were selected on PYE-Rifampcin-Tetracycline (PRT) plates. The respective antibiotics were used because SBI/II$^-$ is resistant to rifampicin and the pRPS-MCS3 plasmid confers resistance to tetracycline. Hence, the PRT selection resulted in the growth of only the transconjugants and not the cells (both *E. coli* and *R. capsulatus*) that failed to mate. Approximately, 300-500 colonies were observed on these plates as a result of matings carried out with 1 ml of the *R. capsulatus* SBI/II$^-$ culture (grown to stationery phase).

Furthermore, the same mating procedure was carried out with several control strains to observe the phenotypes conferred by different RubisCO genes and to ensure that the mating procedure was carried out efficiently. The three positive controls utilized in this study were *R. capsulatus* SBI/II$^-$ complemented with the wild-type *Synechococcus* PCC6301 *rbcLS* genes (form I RubisCO), wild-type *R. palustris cbbM* gene (form II

RubisCO) and the mutant *R. palustris cbbM* gene encoding the I165V substitution, because they have been confirmed to support CO_2-dependent photoautotrophic growth (3, 1). On the other hand, the *R. palustris cbbM* genes containing either I165A or I165T mutations, the *C. tepidum* Mut*CDΔβH* gene, and the *Synechococcus* PCC6301 mutant gene encoding a large-subunit D103V substitution were all used as negative controls because none of them support growth under photoautotrophic conditions (1, 3). Finally, SBI/II$^-$ without any complemented genes was also used as a control to ensure that the knockout strain truly does not support CO_2-dependent growth.

The eight control strains were plated on PYE-Rifampicin, PRT, malate and minimal media plates. As expected, all eight strains were observed to be growing on the PYE-Rifampicin plate (Figure 5). SBI/II$^-$ is not resistant to tetracycline; thus, the unmated strain did not grow on the PRT plate (Figure 5). All the other strains that went through successful mating were growing on these plates, because they took up the pRPS-MCS3 plasmid (carrying the gene for conferring tetracycline resistance). Hence, it was confirmed that the mating procedure and selection on PRT plates were both carried out appropriately.

Following the confirmation of successful mating, all control strains were streaked on malate and minimal (containing no organic carbon) media plates and placed in front of lights. This was carried out in order to observe the growth phenotype of the strains under photo-heterotrophic (malate) and photoautotrophic (minimal media) growth conditions. Results obtained showed that the wild-type and *rbcL*-D103V mutant *Synechococcus* PCC6301 *rbcLS* genes, wild-type *R. palustris cbbM*, and the I165V-mutant *R. palustris cbbM* genes were capable of complementing R. capsulatus SB I/II$^-$ for growth in the

presence of light (energy source) and malate as the carbon source (Figure 6). Owing to severe defects in the catalytic properties of the corresponding RubisCO enzymes, the I165A and I165T mutant genes of *R. palustris cbbM* genes could barely support growth of *R. capsulatus* SB I/II$^-$ under the same conditions (1). Growth on minimal media requires utilization of light as energy source and RubisCO-dependent CO_2 fixation as the only means to procure carbon. Only the wild-type *Synechococcus* PCC6301 *rbcLS* genes, wild-type *R. palustris cbbM*, and I165V-mutant *R. palustris cbbM* genes could confer minimal-media growth competency to *R. capsulatus* SB I/II$^-$ (Figure 6). The recovered suppressors were compared with these strains on minimal media, in order to confirm that the randomly-introduced mutations were indeed effective in suppressing the original negative mutations.

Previous work in our laboratory had utilized the random mutagenesis and bioselection procedure to isolate both favorable and non-favorable mutants of RubisCO (3). Hence, it was hypothesized that the same procedure might presumably yield a mutant CtRLP protein that would support CO_2 dependent growth, if it required the presence of only one or two additional mutations, in addition to those introduced already (1). However, such a mutant could not be isolated at the end of this study. The same procedure was carried out several times with no sign of a mutant that supported growth in an anaerobic setting in the presence of 5-10% CO_2. After repeated unsuccessful attempts, it was hypothesized that a higher CO_2 concentration may facilitate the recovery of RubisCO function. Hence the same mutagenesis procedure was carried out and the cells were allowed to grow in an anaerobic setting with 20% atmospheric CO_2. However, upon repeating the experiment multiple times, no colonies could be observed on minimal

media. The procedure utilized in this study allowed a limited number of single base pair mutations within the gene. It is possible that such base pair mutations alone could not yield an RLP that would support photoautotrophic growth. Perhaps other structural changes in the protein are required to yield an RLP molecule that would support CO_2 dependent growth. Multiple approaches may be required in order to obtain RubisCO function from an RLP.

The second focus of this study was the form II RubisCO from *R. palustris*. Mutant genes that encode form II RubisCO with the I165A and I165T substitutions were the target of the random mutagenesis procedure. As hypothesized, it was indeed possible to select for a single suppressor mutation (from multiple rounds of bioselection) that acts to overcome the negative effect of the original I165A substitution. The suppressor in this case was a "pseudo-revertant" because the alanine at position 165 was changed to valine (A165V). Valine at residue 165 has already been confirmed to support photoautotrophic growth (Figure 6) (1). At the end of this study, four pseudo-revertants were found that resulted from a single base-pair mutation (C→T), which would change the alanine residue at position 165 to valine. These pseudo-revertants were named RPE, RPF, RPG and RPJ (Figure 7).

Originally, six other strains were also recovered after bioselection based on their ability to support photoautotrophic growth of R. capsulatus SB I/II$^-$. They were named RPA, RPC, RPD, RPH, RPI and SS3. These colonies were grown in rich PYE media and the plasmid was sent for sequencing after purification. Sequencing results showed that these strains all contained only the original I165A substitution with no other mutations except some in the N-terminus. The same N-terminal mutations were also seen in the four

pseudo-revertant genes. It was unclear if these N-terminal substitutions played any role in supporting growth under photoautotrophic conditions. To verify the results, the purified plasmid from each of the ten colonies was re-mated into *R. capsulatus* SBI/II⁻. All the strains were plated on malate and minimal media plates to check for their ability to confer photo-heterotrophic and photoautotrophic growth. It was observed that only the four strains that contained the I165V substitution were able to grow under photoautotrophic conditions (Figure 7). These results confirmed that the N-terminal substitutions did not play a role in supporting growth under photoautotrophic conditions. It also suggested that the six plasmids that failed to complement growth upon re-mating, must have shown false growth beforehand or must have been mixed in the host cells with multiple plasmids. It is possible that these plasmids without any additional mutations were the ones that got sequenced.

There are some limitations that could explain why no true suppressors of I165A and I165T were obtained at the end of this study. Firstly, it is possible that no suppressors of I165A and I165T were obtained because a higher sampling number is required in order to obtain such suppressors. This could be the same reason why no strains with a true reversion from threonine back to isoleucine at position 165 were obtained, as expected from various possible single-base changes at the same codon (Table 2). Secondly, it is also possible that only isoluecine and valine at position 165 are able to confer functionality to the enzyme.

One factor that needs to be taken into consideration is the N-terminal substitutions that were observed in the sequencing results of recovered mutants. Upon analysis of the *cbbM* gene sequence it was discovered that the N-terminal substitutions within the mutant

gene were a result of recombination. This recombination occurred between the N-terminal region of the mutant gene and its homologous region that is present within the expression vector used for this study, pRPS-MCS3 (Figure 3). The first thing to do would be to devise a method to remove this homologous region from the plasmid or to utilize another expression vector. This would ensure that no recombination takes place and the gene of interest is inserted within the plasmid strictly via the ligation procedure and not via recombination. Previous successful suppressor mutant isolations employed the *Synechococcus rbcLS* genes with the same vector used here (3). Of course the *Synechococcus* genes contain no homologous sequences to *cbbM* and it was thus not realized that the expression vector would create a problem for mutagenesis and selection experiments employing *cbbM* genes.

Additionally, it is possible that during the mating procedure *R. capsulatus* takes up more than one pRPS-MCS3 plasmid. Some plasmids may contain a mutated gene that confers photoautotrophic growth and some do not. In this study, all *R. capsulatus* colonies sent for sequencing were amplified in rich CSOC media. It is highly possible that the colony contained a 1/10 ratio of plasmids with a mutant gene that allows photoautotrophic growth to those that do not. If that was the case, after growth in rich media there would be a higher concentration of plasmids without the mutated gene than those that do. Hence, when the plasmid was purified it is highly probable that a plasmid with no mutated gene was the product and so no new substitutions were observed in the sequencing results. This could be the reason why six of the ten original strains that were thought to be suppressors just showed the original I165A substitution. To resolve this problem it is necessary to grow all mated *R. capsulatus* colonies in minimal media prior

to plasmid purification and sequencing. This step would presumably enrich for the plasmid with the gene that supports photoautotrophic growth. With the alteration of this step, there is a higher probability that a plasmid conferring photoautotrophic growth will be sent for sequencing. Therefore, there will be a better chance of recovering a true suppressor.

In summary, this study has been successful in adopting the previously described bioselection procedure for recovery of favorable substitutions in *R. palustris* form II RubisCO, although the recovered mutations resulted only in pseudoreversions. The suggested alterations in the procedures utilized would perhaps eliminate some of the limitations identified here and potentially enhance the chances of recovering a true second-site suppressors. Additionally this study was able to standardize the random mutagenesis and bioselection procedure with the CtRLP, although no favorable mutation could be recovered. Suppressor recovery would presumably allow the discovery of additional residues in RubisCO that contribute to function and hence, shed light upon the structure-functional relationships of the enzyme.

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8. Tabita, F. R., Hanson, T. E., Li, H., Satagopan, S., Singh, J. & Chan, S. (2007). Function, structure, and evolution of the RubisCO-like proteins and their RubisCO homologs. *Microbiology and Molecular Biology Reviews,* **71**, 576-599.

FIG. 1. Calvin Benson-Bassham (CBB) pathway. The first step of the CBB cycle catalyzed by RubisCO requires the fixation of 3 CO_2 molecules in order to convert 3 RuBP (C_5) molecules to 6 molecules of 3- phosphoglycerate (PGA) (C_3). 6 PGA (C_3) molecules are converted to 6 molecules of 1,3 phosphoglycerate (DPGA, C_3) which requires 6 ATP molecules. 6 molecules of DPGA are converted to 6 glyceraldehyde 3-phosphate (GAP, C_3) which requires 6 NADPH molecules. PRK catalyzes the last step to convert 5 GAP (C_3) molecules back to 3 RuBP (C_5) molecules which serve as the substrate to repeat the process. This last step requires 3 ATP molecules (2).

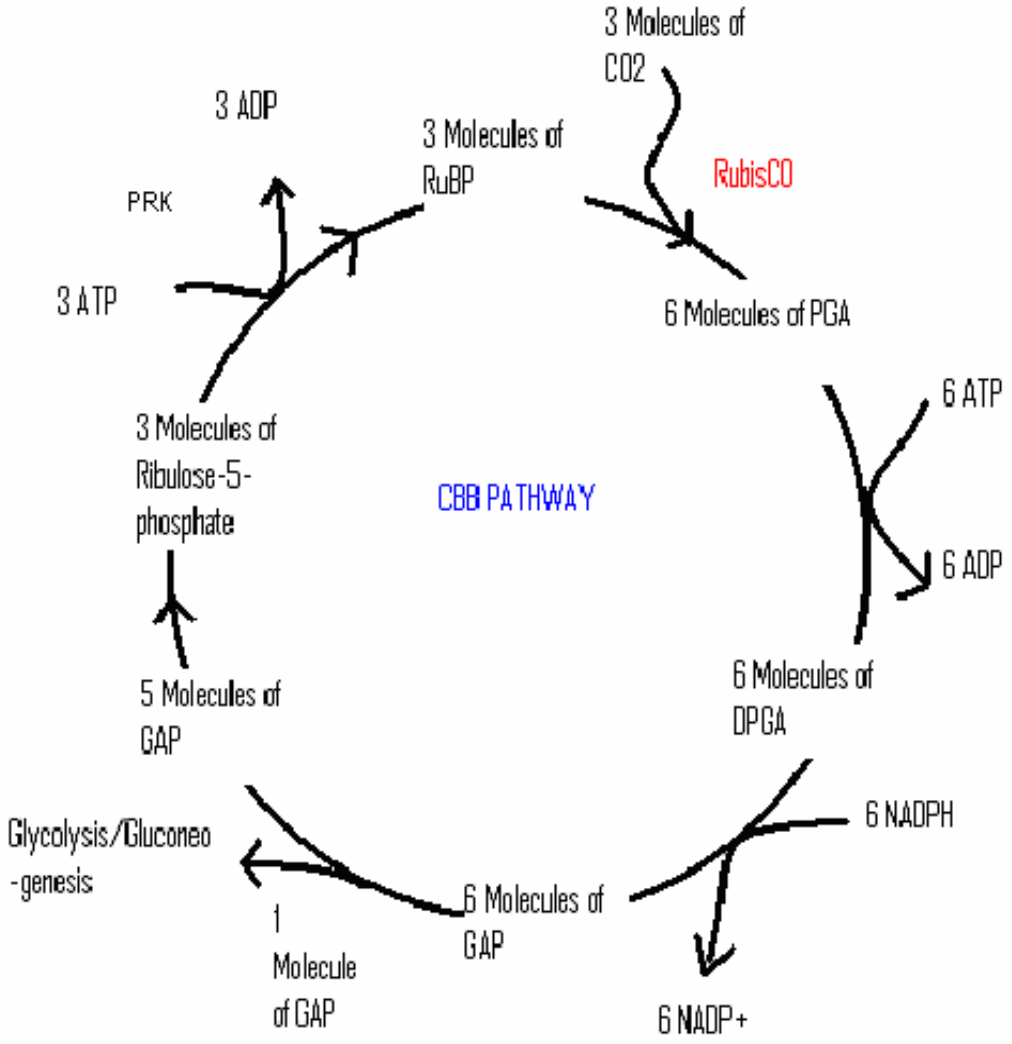

3 Molecules of CO2
3 ADP
3 Molecules of RuBP
RubisCO
PRK
3 ATP
6 Molecules of PGA
3 Molecules of Ribulose-5-phosphate
6 ATP
CBB PATHWAY
6 ADP
5 Molecules of GAP
6 Molecules of DPGA
Glycolysis/Gluconeo-genesis
6 NADPH
1 Molecule of GAP
6 Molecules of GAP
6 NADP+

FIG. 2. The pRPS-MCS3 plasmid. The host range expression plasmid pRPS-MCS3 contains the *Rhodospirillum rubrum* RubisCO (*cbbM*) gene promoter and the transcriptional activator gene, *cbbR*, to drive the expression of RLP and form II RubisCO genes cloned into the multiple cloning site of the plasmid. The plasmid contains a *lacZα* gene for blue/white screening. Following transformation into *E. coli*, colonies containing the vector with the gene of interest would be white on X-gal plates since the gene would disrupt the *lacZα* gene in the vector. The vector also contains a tetracycline-resistance gene (Tet). The antibiotic resistance marker would only allow colonies that took up the vector to survive. Additionally, the vector possesses a partial sequence of the *R. rubrum cbbm* gene (3).

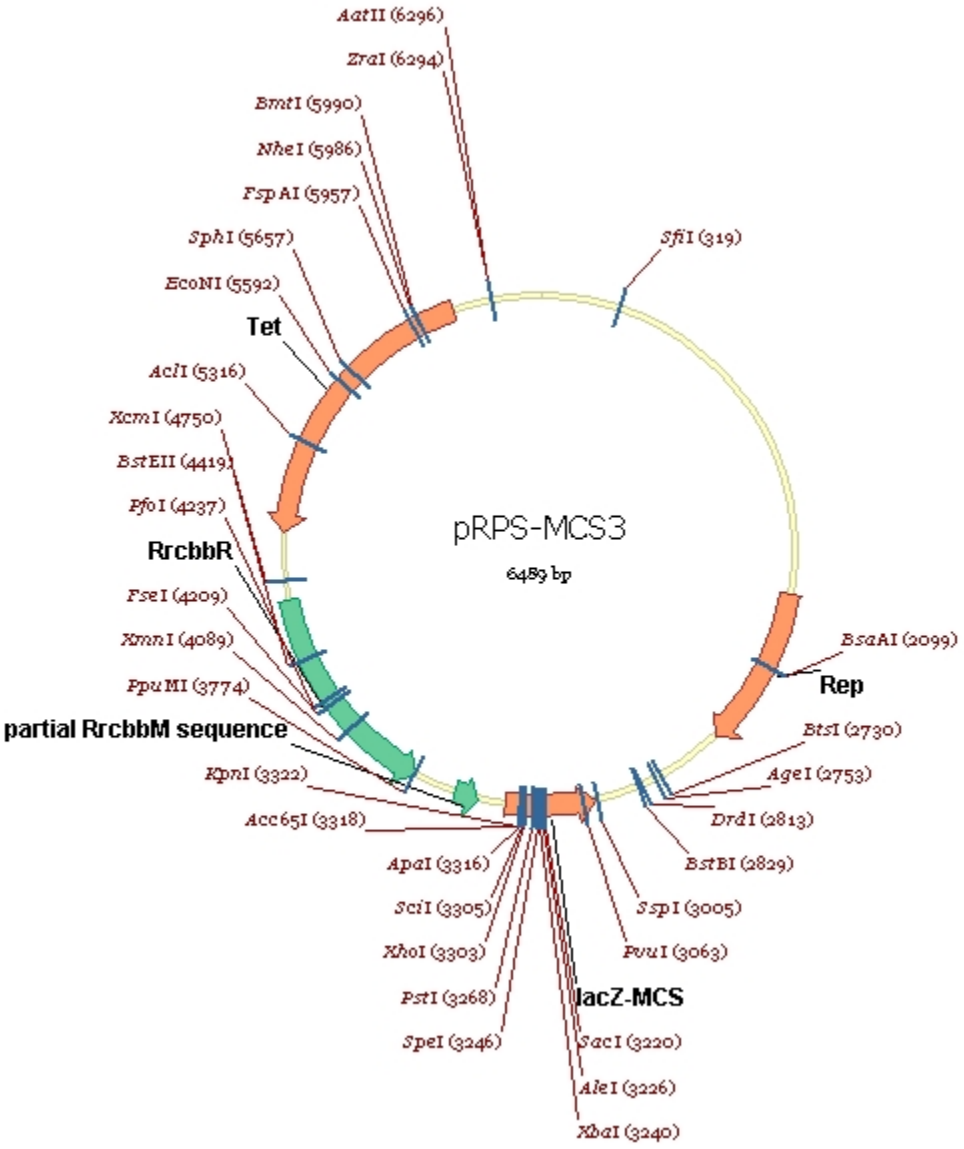

AatII (6296)
ZraI (6294)
BmtI (5990)
NheI (5986)
FspAI (5957)
SphI (5657)
EcoNI (5592)
Tet
AclI (5316)
XcmI (4750)
BstEII (4419)
Pfo1 (4237)
RrcbbR
FseI (4209)
XmnI (4089)
PpuMI (3774)
partial RrcbbM sequence
KpnI (3322)
Acc65I (3318)
ApaI (3316)
SciI (3305)
XhoI (3303)
PstI (3268)
SpeI (3246)
SfiI (319)
pRPS-MCS3
6489 bp
BsaAI (2099)
Rep
BtsI (2730)
AgeI (2753)
DrdI (2813)
BstBI (2829)
SspI (3005)
PvuI (3063)
lacZ-MCS
SacI (3220)
AleI (3226)
XbaI (3240)

FIG 3. Triparental Mating. *R. capsulatus* knockout strain, SBI/II$^-$, had been previously constructed by deleting both forms I and II RubisCO found in wild-type *R. capsulatus* (5). Hence, *R. capsulatus* SBI/II$^-$ is unable to carry out CO_2 dependent growth and has previously been successfully used in a random mutagenesis and bioselection system (3). In this system, randomly mutagenized RLP and mutant RubisCO genes were expressed in *R. capsulatus* SBI/II$^-$. Gene expression in *R. capsulatus* SBI/II$^-$ was accomplished by mating that strain with *E. coli* Top 10 cells containing the pRPS-MCS3 plasmid carrying the randomly mutagenized genes. The mating also requires *E. coli* cells containing the plasmid pRK-2013, which facilitates bacterial conjugation (3). All three types of cells for mating were grown on plates and then inoculated into liquid cultures. Both of these steps were performed with the addition of appropriate antibiotics under optimum conditions. Following the mating procedure, the mating mixture was placed in a concentrated area on a PYE plate to promote conjugation. Finally, the conjugated cells were plated on PRT plates for selection of successfully mated *R. capsulatus* cells and then onto minimal media plates to select for suppressors that confer CO_2-dependent growth.

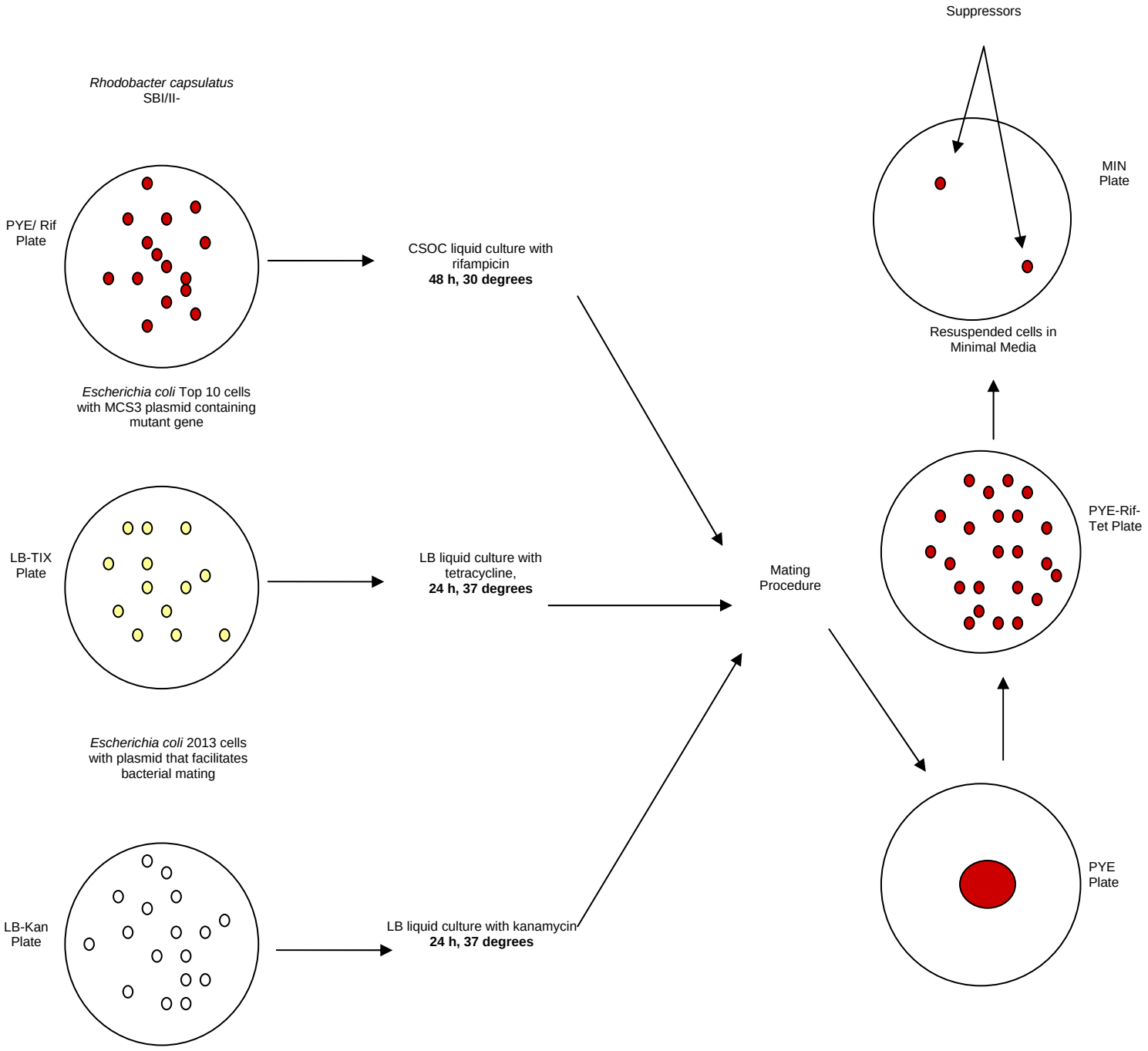
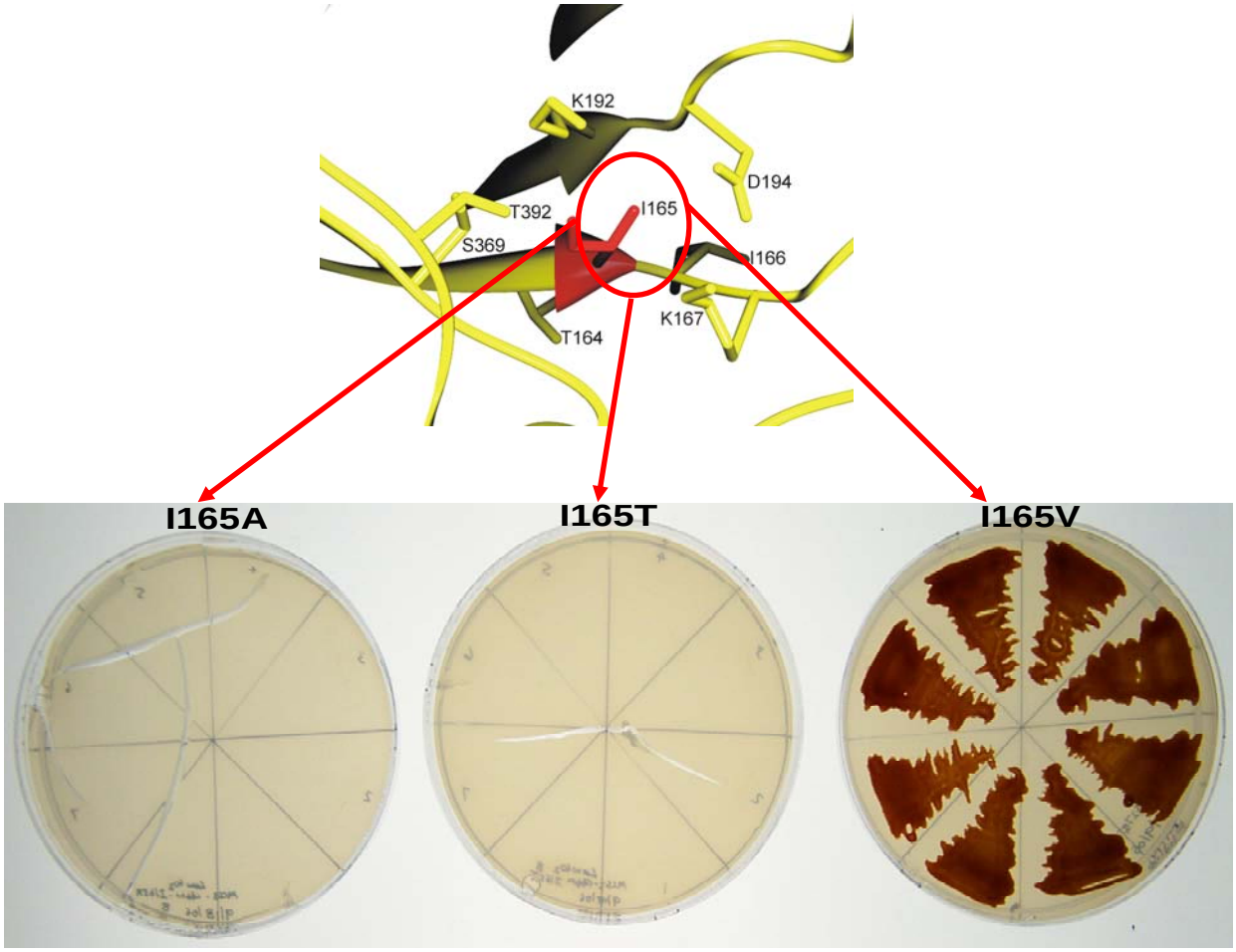

Suppressors
Rhodobacter capsulatus
SBI/II-
PYE/ Rif
Plate
CSOC liquid culture with
rifampicin
48 h, 30 degrees
MIN
Plate
Resuspended cells in
Minimal Media
Escherichia coli Top 10 cells
with MCS3 plasmid containing
mutant gene
LB-TIX
Plate
LB liquid culture with
tetracycline,
24 h, 37 degrees
Mating
Procedure
PYE-Rif-
Tet Plate
Escherichia coli 2013 cells
with plasmid that facilitates
bacterial mating
LB-Kan
Plate
LB liquid culture with kanamycin
24 h, 37 degrees
PYE
Plate

FIG 4. Growth phenotypes of mutant enzymes, I165A, I165T and I165V. Upon sequence comparison of RubisCO forms I, II and III, it was observed that residue 165 of *Rhodopsuedomonas palustris* form II RubisCO was a residue that was different among the three forms of RubisCO (threonine in form I, isoleucine in form II and valine in form III). In order to examine the importance of this residue, isoleucine in the *R. palustris* form II RubisCO was substituted to alanine (a neutral substitution), threonine (resembling form I RubisCO) and valine (resembling form III RubisCO) via site-directed mutagenesis. Results showed that mutant enzymes, I165A and I165T were negative mutants that did not catalyze the CO_2 fixation reaction when placed in anaerobic jars containing 10% CO_2. On the other hand, mutant enzyme, I165V was able to support photoautotrophic growth under the same conditions (1).

R. palustris form II (*cbbM*)



Neutral substitution

Substitution that resembles Form I RubisCO

Substitution that resembles Form II RubisCO

Kinetic Properties of Form II Wild-Type RubisCO and mutant enzymes,

I165A, I165T and I165V

Enzyme	k_{cat} (s-1)	Specificity ($\Omega = V_cK_o/V_oK_c$)	Km (CO_2) (μM)	Km (O_2) (μM)	K_o/K_c	V_c/V_o	K_m(RuBP) (μM)
Wild type	**4.4 ± 0.2**	**12 ± 1**	**245 ± 64**	**190 ± 11**	**0.8**	**15**	**12 ± 2**
I165A	**0.9 ± 0.2**	**6 ± 2**	**520 ± 96**	**189 ± 43**	**0.4**	**15**	**10 ± 2**
I165T	**0.8 ± 0.1**	**11 ± 2**	**697 ± 250**	**2883 ± 283**	**4.1**	**3**	**38 ± 1**
I165V	**1.5 ± 0.3**	**4 ± 1**	**300 ± 35**	**206 ± 66**	**0.7**	**6**	**29 ± 2**

Values with means ± SD (n-1) are from at least 3 sets of duplicate measurements

Table 1. Kinetic properties of purified I165A, I165T and I165V mutant enzymes and wild-type *R. palustris* form II RubisCO (7).

I165A & I165T are good candidates for "bioselection"

Ile (ATC) → Ala (GCC) → one-base change → Val (GTC)
→ Thr (ACC) → one-base change → Ile (ATC)
→ Val (GTC)

Ala (GCC)			Thr (ACC)		
ACC	**GAC**	**GCA**	**TCC**	**AAC**	**ACA**
Thr	**Asp**	**Ala**	**Ser**	**Asn**	**Thr**
TCC	**GTC**	**GCT**	**GCC**	**ATC**	**ACT**
Ser	**Val**	**Ala**	**Ala**	**Ile**	**Thr**
CCC	**GGC**	**GCG**	**CCC**	**AGC**	**ACG**
Pro	**Gly**	**Ala**	**Pro**	**Ser**	**Thr**

Table 2. Single base pair mutations in the codons for Alanine and Threonine and the variety of amino acids encoded by these mutations (7)

FIG 5. Control Mating Experiments. All control strains grew on the PYE-Rifampicin plate since all the genes were complemented into *R. capsulatus* which is resistant to rifampicin. Only the *R. capsulatus* SBI/II^{-} strain without any genes complemented into it did not grow on the PYE-RIF/TET plate because it did not contain the pRPS-MCS3 plasmid which contains the resistance gene for tetracycline. This was expected because only the strains that went through successful mating took up the pRPS-MCS3 plasmid which contained the tetracycline resistance gene.

Control Mating Experiments

PYE-RIF

PYE-RIF/TET

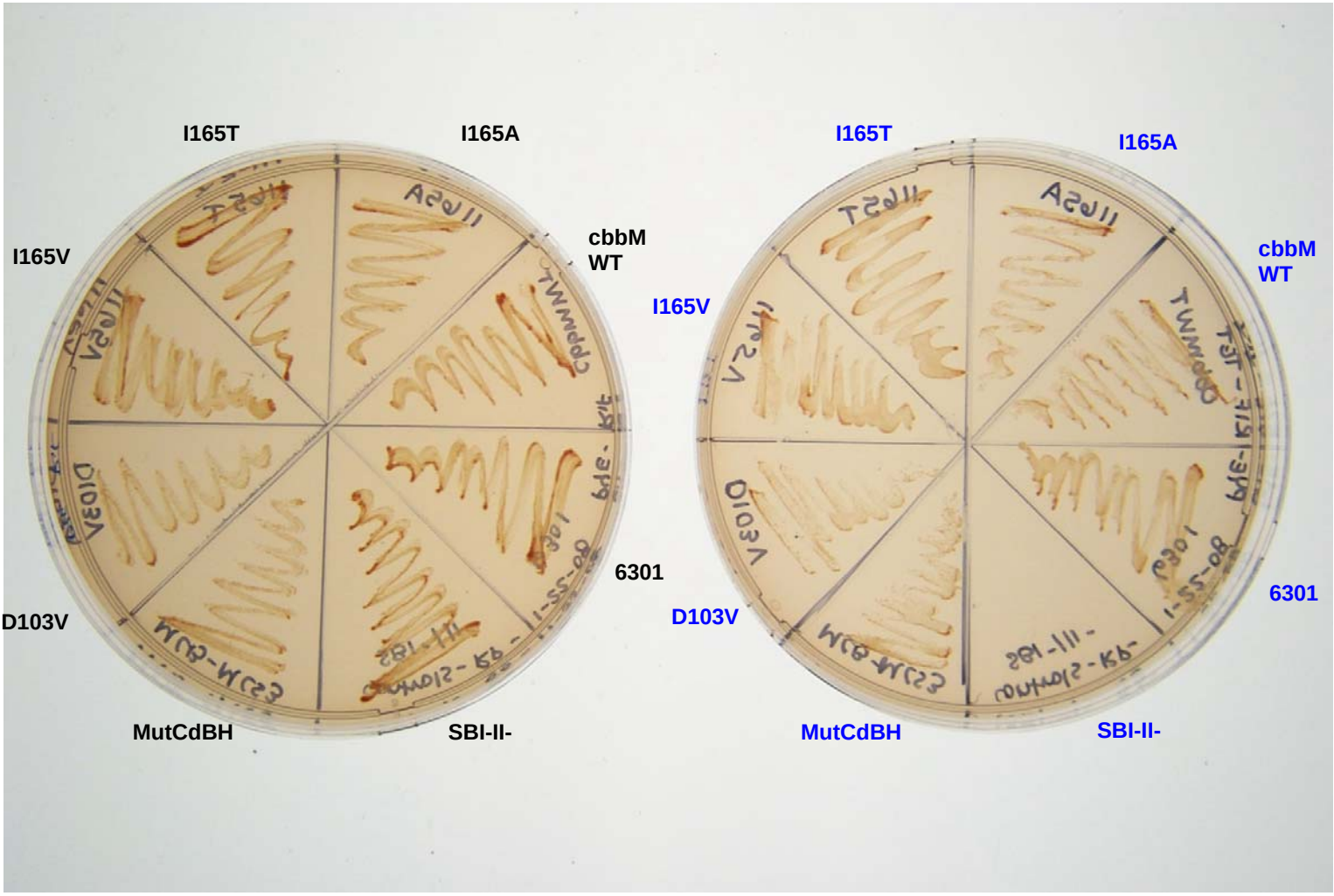
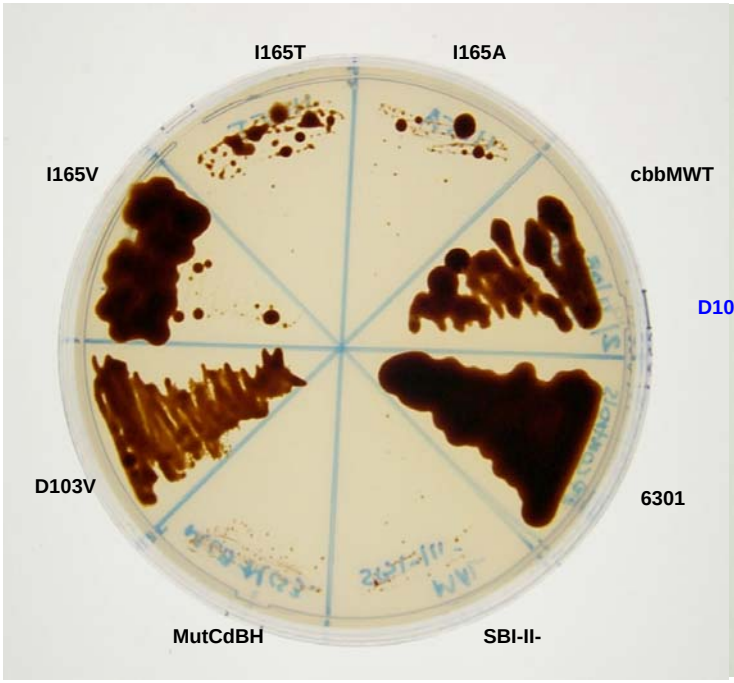


FIG 6. Control Mating Experiments. Malate Media was used to observe growth phenotypes of control strains under photo-heterotrophic growth conditions. Minimal Media was used to observe growth phenotypes under photoautotrophic conditions. Results obtained showed that *R. capsulatus* SBI/II$^-$ strain complemented with the wild-type *Synechococcus* PCC6301 *rbcLS* gene, wild-type *R. palustris cbbM* gene, *R. palustris cbbM* gene containing the I165V substitution and *Synechococcus* PCC6301 mutant *rbcLS* gene containing a D103V substitution were capable of photo-heterotrophic growth. Additionally, *R. capsulatus* SBI/II$^-$ strain complemented with the *R. palustris cbbM* gene containing the I165A and I165T substitutions showed less growth under the same conditions. On the other hand, *R. capsulatus* SBI/II$^-$ strain complemented with the *Synechococcus* PCC6301 *rbcLS* genes, wild-type *R. palustris cbbM* gene and *R. palustris cbbM* gene containing the I165V substitution were the only strains that conferred growth under photoautotrophic conditions.

Control Mating Experiment

Malate

Minimal



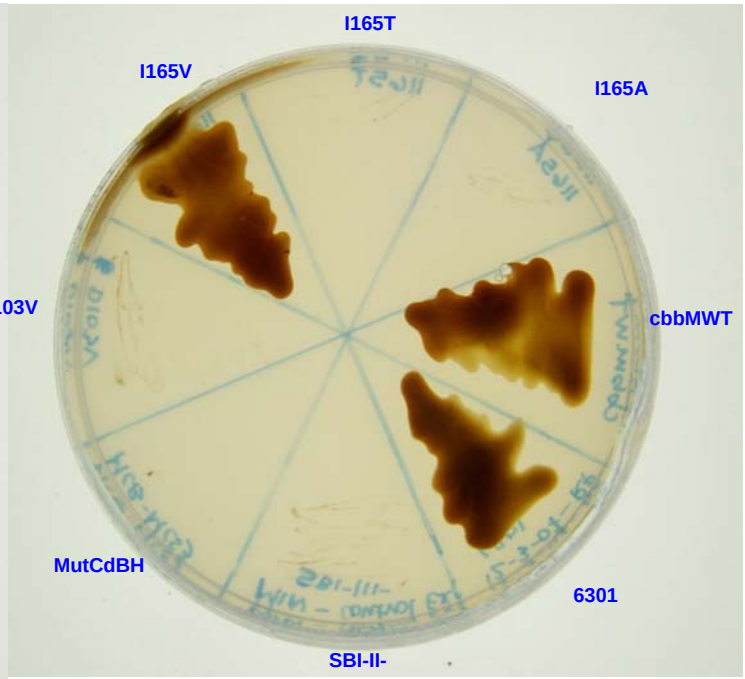
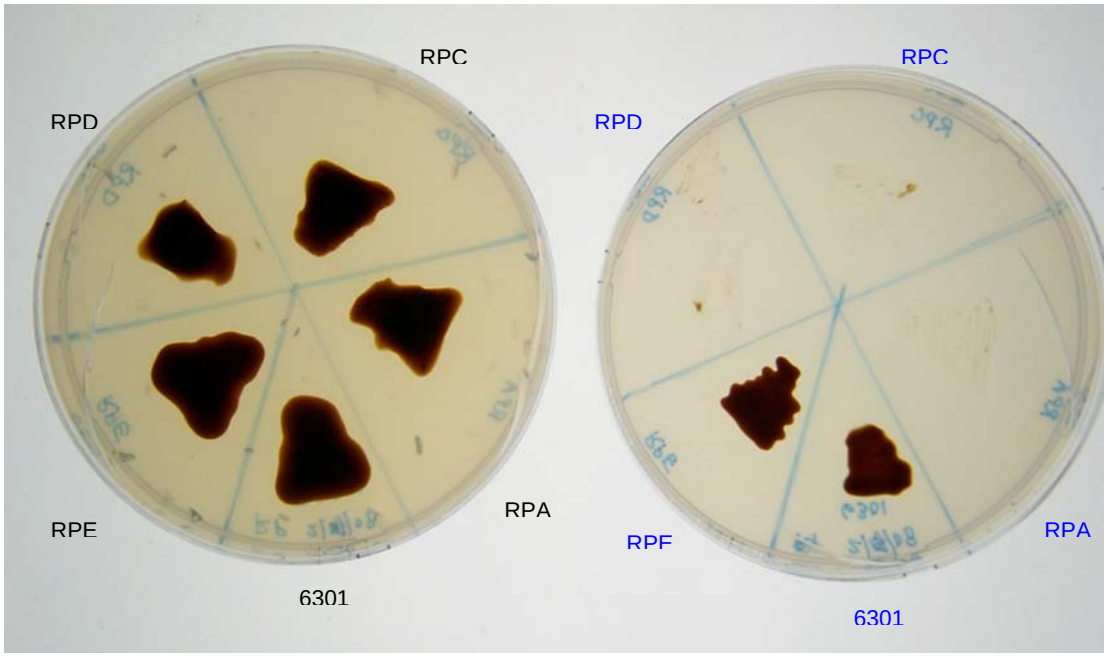


FIG 7. Pseudo-revertant confirmation. Ten strains: RPA, RPC, RPD, RPE, RPF, RPG, RPH, RPI, RPJ and SS3 were originally thought to be suppressors because they appeared to be growing under photoautotrophic conditions. However, sequencing results showed that RPA, RPC, RPD, RPH, RPI and SS3 contained only the original I165A substitution with no other mutations except some in the N-terminus. On the other hand, RPE, RPF, RPG and RPJ contained the same N-terminal mutations along with the "pseudo-reversion" from alanine to valine at position 165. It was unclear if these N-terminal substitutions played any role in supporting growth under photoautotrophic conditions. To verify the results, the purified plasmid from each of the ten colonies was re-mated into *R. capsulatus* SBI/II^{-}. All the strains were plated on malate and minimal media plates to check for their ability to confer photo-heterotrophic and photoautotrophic growth. It was observed that only the four strains that contained the I165V substitution were able to grow under photoautotrophic conditions. This confirmed the fact that the N-terminal substitutions did not play a role in supporting growth under photoautotrophic conditions. It also suggested that the six strains that did not show growth must have shown false growth beforehand or must have been mixed in the host cells with multiple plasmids. It is possible that these plasmids without any additional mutations were the ones that got sequenced.

Pseudo-Revertant
Confirmation

Malate

Minimal



Malate

Minimal